Engineering de novo reciprocal chromosomal translocations associated with *Mll* to replicate primary events of human cancer

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Summary

The etiology of human tumors often involves chromosomal translocations. Models that emulate translocations are essential to understanding the determinants of frank malignancy, those dictating the restriction of translocations to specific lineages, and as a basis for development of rational therapeutic methods. We demonstrate that developmentally regulated Cre-*loxP*-mediated interchromosomal recombination between the *Mll* gene, whose human counterpart is involved in a spectrum of leukemias, and the *Enl* gene creates reciprocal chromosomal translocations that cause myeloid tumors. There is a rapid onset and high penetrance of leukemogenesis in these translocator mice, and high proportions of cells carrying chromosomal translocations can be found in bone marrow as early as 12 days after birth. This de novo strategy is a direct recapitulation of naturally occurring human cancer-associated translocations.

Introduction

The etiology of tumors often involves chromosomal translocations in hematopoietic malignancies, sarcomas, and epithelial tumors, and these can either activate protooncogenes or create novel fusion genes that function in tumorigenesis (Rabbitts, 1994). These translocation products act at various levels in afflicted cells, although the translocation genes always encode intracellular proteins located in either the cytoplasm or nuclei. The recurrent chromosomal translocations in hematopoietic malignancies and in sarcomas generally display a tropism in respect of the cell type in which they are found, for instance, the *MLL* gene fusion subtypes being restricted to either lymphoid or myeloid malignancies (Downing and Shannon, 2002; Look, 1997; Rowley, 1998). The *MLL* gene is a frequent site of chromosomal translocations in human leukemias (Corral et al., 1993; Djabali et al., 1992; Domer et al., 1993; Gu et al., 1992; Thirman et al., 1993; Tkachuk et al., 1992), affecting over 30 different chromosomes and resulting in many leukemia-associated *MLL*-gene fusions (Ayton and Cleary, 2001; Collins and Rabbitts, 2002). Different fusions distinguish distinct leukemias, for instance, the *MLL-AF9* fusion is almost exclusively in myeloid leukemias while *MLL-AF4* is confined to B lymphoid leukemias (Gu et al., 1992; Rowley, 1998). Others, such as *MLL-ENL*, can be found in myeloid and lymphoid leukemias (Ayton and Cleary, 2001).

Mouse models which mimic the effects of chromosomal translocations, or which recapitulate these events, are essential to understanding the determinants of frank malignancy and those dictating the restriction of translocations to specific lineages. In addition, with advancement of efforts to devise new reagents which can interfere with protein function, mouse models of human cancer are crucial to developing and testing these rationally devised therapeutic approaches prior to application in patients. Finally, mouse models of human cancer will be needed for in vivo testing of new compounds, which may have promising biochemical properties, in order to accelerate the translation of these into clinical use. Mouse cancer models involving chromosomal translocation genes have been either gain-of-function transgenic models (Adams et al., 1999; Pandolfi, 1998), retroviral transduction models, or translocation mimics in which a gene fusion is created by homologous recombination into an endogenous target gene using embryonic stem cells (ES cells) (Corral et al., 1996). For instance, the consequences of *MLL*-associated translocations have been modeled in mice using retroviral transduction of bone marrow progenitors (Lavau et al., 1997; Schulte et al., 2002; Slany et al., 1998) or by homologous recombination knockin of fusion genes (Corral

SIGNIFICANCE

Mouse models of human cancers are important for understanding determinants of overt disease and for "preclinical" development of rational therapeutic strategies. Chromosomal translocations underlie many human leukemias, sarcomas, and epithelial tumors. There are several approaches modeling the effects of chromosomal translocations in mice but none fully recapitulate human translocations and none generate both reciprocal products. We describe a system by which chromosomal translocations occur de novo during mouse development and cause tumors carrying both reciprocal translocated chromosomes. This de novo model mirrors the situation found in humans and provides the framework for design of any chromosomal translocation in mice. In this model, we have studied the *Mll* gene fusion with *Enl* and found a high penetrance and rapid onset of tumors.

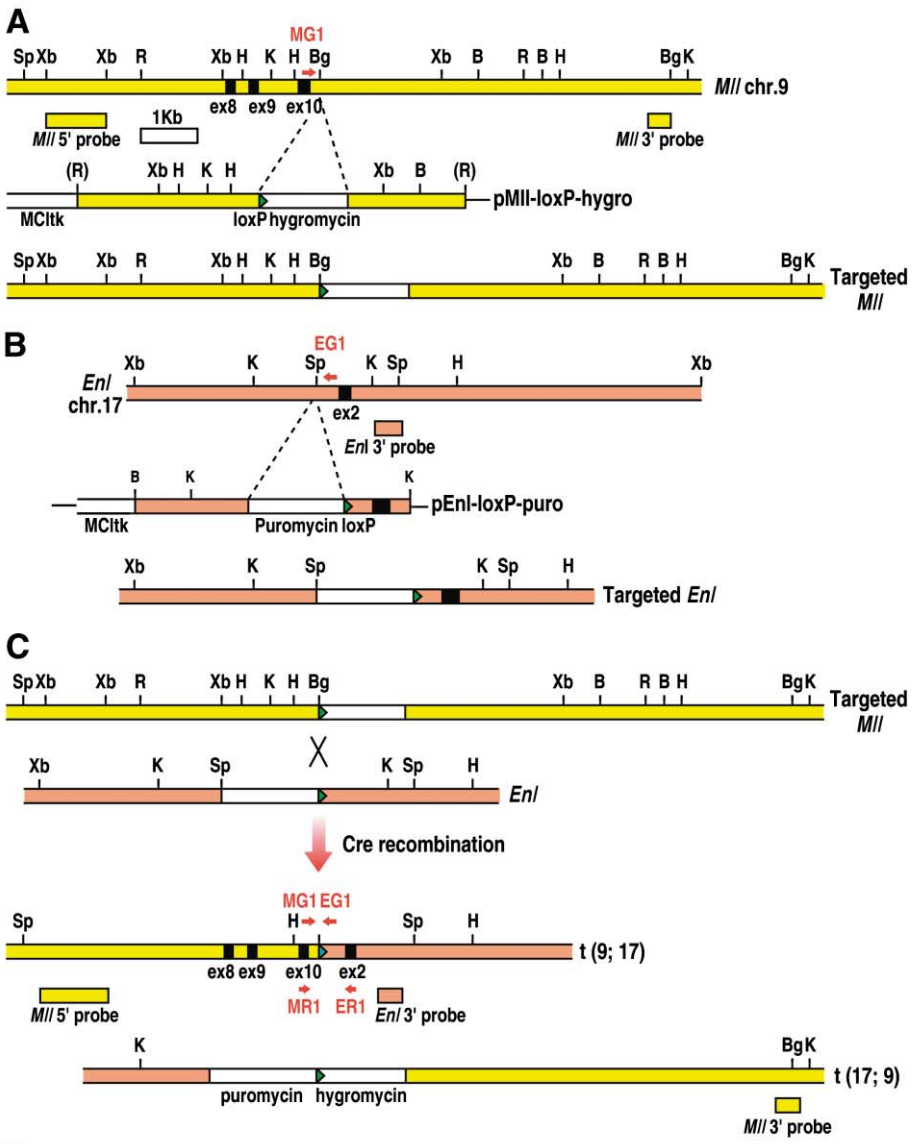

Figure 1. Targeting and recombination strategy for *Mll* and *Enl* genes

LoxP recombination sites were introduced into the mouse *Mll* (Collins et al., 2000) and *Enl* genes using gene targeting in ES cells. The targeted cells were injected into blastocysts, and chimaeric mice that carried the modified alleles were derived. These were bred to obtain germline transmission and subsequently interbred with each other and with a line in which Cre recombinase is expressed from the *Lmo2* gene.

A: Map of the mouse *Mll* gene indicating three exons of the gene and the BglII restriction site at which was cloned a *loxP* cassette with a hygromycin-selectable marker (Johnson et al., 1995). The pMll-loxP-hygro targeting vector is shown in the middle line and the organization of the targeted allele in the bottom line, showing that the *loxP* site is downstream of exon 10 of *Mll*.

B: The mouse *Enl* gene indicating exon 2 and the SphI restriction site at which was cloned a *loxP* cassette with a puromycin-selectable marker (Linnell et al., 2001). The top line is the germline map, the middle is the pEnl-loxP-puro targeting vector, and the bottom is the structure of the targeted allele.

C: Cells or mice with both the *Mll* (chromosome 9) and *Enl* (chromosome 17) *loxP* alleles can undergo Cre-dependent interchromosomal reciprocal translocation. The derivative t(9.17) and t(17;9) are diagrammatically shown in the 3rd and 4th lines, respectively.

D and **E:** To verify the ability of the *Mll* and *Enl* genes to participate in interchromosomal translocations, Cre recombinase was transiently expressed in ES cell lines carrying both *loxP* alleles. Genomic DNA or mRNA were prepared for genomic PCR (**D**) or RT-PCR (**E**) with *Mll*- and *Enl*-specific primers. For the genomic PCR, primers MG1 (from *Mll*) and EG1 (from *Enl*) allowed amplification of a 430 bp fragment, the sequence of which comprised the junction of the *Mll* chromosome, the *loxP* site, and the *Enl* chromosome (**D**). RT-PCR was carried out with RNA from the cells using an *Mll* exon 10 primer (MR1) and an *Enl* exon 2 primer (ER1) to determine if the *Mll-Enl* fusion mRNA could be detected. A 390 bp PCR was detected and sequenced, showing the in-frame fusion of *Mll* and *Enl* sequences (**E**).

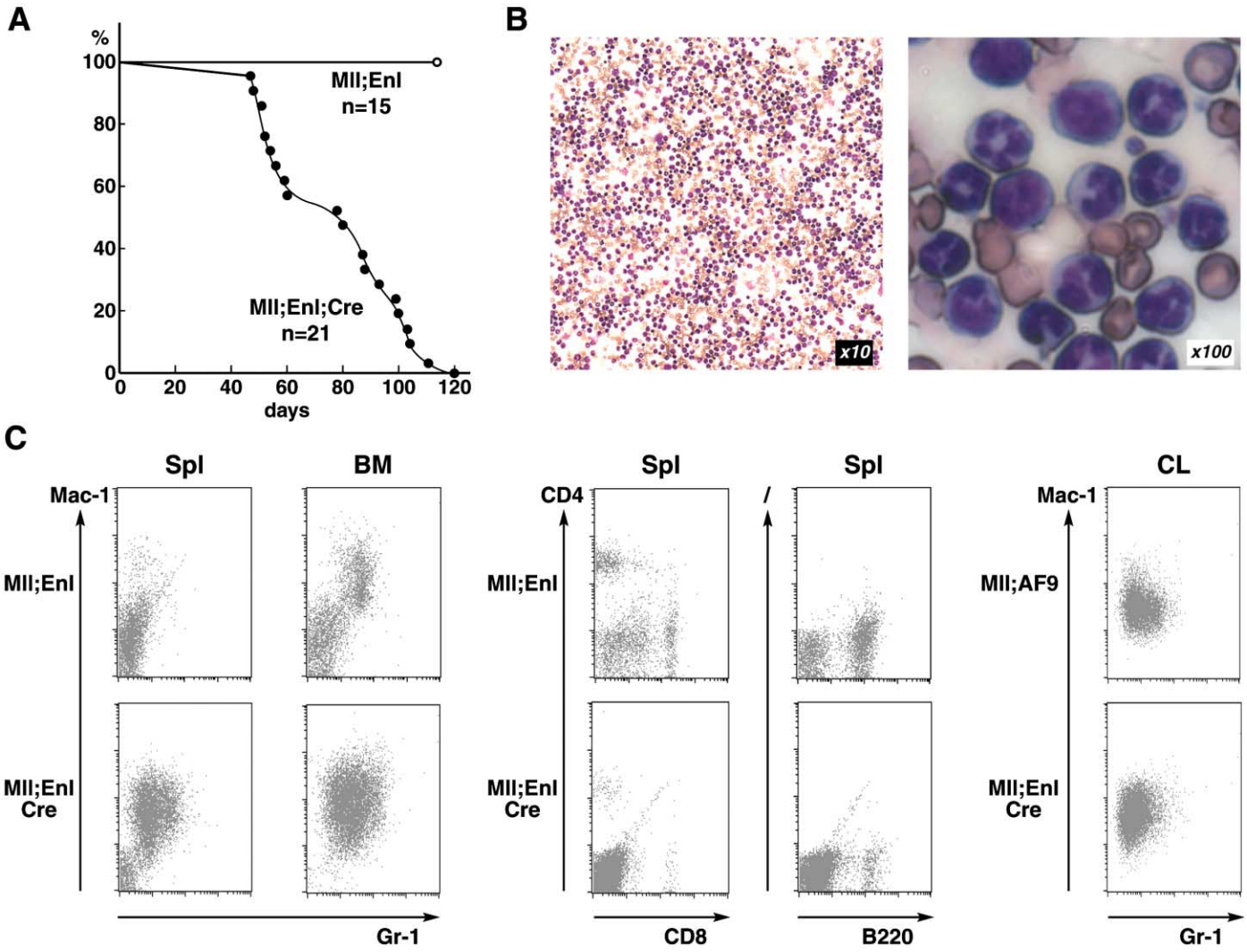

Figure 2. Myeloid leukemia development depends on Cre expression

A cohort of animals (n = 21) was generated which had the genotype *Mll-loxP; Enl-loxP; Cre* (Mll;Enl;Cre) and a control group (n = 15) with *Mll-loxP; Enl-loxP* alleles (Mll; Enl) but lacking the *Lmo2-Cre* gene. Survival curves are shown in **A**. Mice were carefully observed from birth and post-mortem examination carried out at first signs of ill health. Biopsies of tissues were taken, together with blood smears. Smears were stained with MGG stain, and photographs show the presence of a large number of leukocytes (**B**, X10) and high power shows immature and mature myeloid forms (**B**, X100). Fluorescence activated cell sorter analysis of surface phenotype is shown in **C**. Spleen (spl) and bone marrow (BM) cells were prepared from *Mll-loxP; Enl-loxP; Cre* or from *Mll-loxP; Enl-loxP* mice and stained with fluorescent antibodies as indicated. Antibodies used were FITC-Gr-1 (Ly-6G) plus PE-Mac-1 (CD11b), FITC-CD8a (Ly2), or FITC-B220 (CD45R) plus PE-CD4 (L3T4). The myeloid cells from the *Mll-loxP; Enl-loxP; Cre* mice lack Sca-1 and c-Kit expression. In addition, a cell line (CL) established from a *Mll-loxP; Enl-loxP; Cre* mouse (designated 1) was analyzed with FITC-Gr-1 plus PE-Mac-1 and compared with a cell line established from the *Mll-AF9* knockin mice (Corral et al., 1996).

et al., 1996; Dobson et al., 1999, 2000). In the knockin approach, homologous recombination was used to introduce fusion oncogenes into mice via embryonic stem cells. In this way, a mouse model of the human *MLL-AF9* gene fusion was made in which knockin mice were found to develop an acute myeloid malignancy similar to that found in human patients with the chromosomal translocation t(9;11) (Corral et al., 1996; Dobson et al., 1999). While the knockin approach is a close mimic of natural chromosomal translocations, it is limited by the fact that the homologous recombination event generates a knockin of one allele which is subsequently transferred to all cells of the mouse. Thus the fusion gene will be expressed in all sites where the targeted gene is expressed, for instance as found for the *Mll-AF9* knockin fusion (Corral et al., 1996). In addition, dominant, embryonic lethal effects of knockin alleles have been observed (Okuda et al., 1998; Yergeau et al., 1997). These can be overcome by creating conditional knockin alleles such as the *Aml1-Eto* example, which circumvents the embryonic lethal effects of the fusion and causes AML to arise in mice (Higuchi et al., 2002).

The most ideal concept is the de novo creation of chromosomal translocation in mice, which could potentially be achieved by in vivo recombination systems, such as the Cre-*loxP* system (Smith et al., 1995; van Deursen et al., 1995). This approach

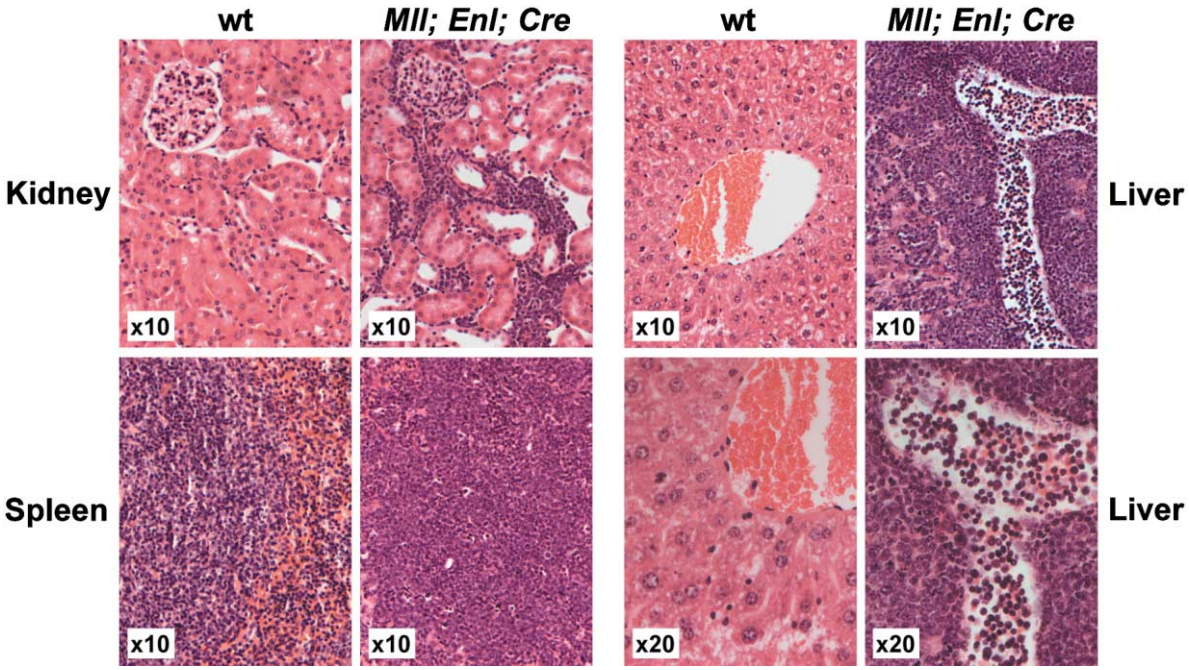

Figure 3. Histology of tissues from *Mll-loxP-Enl-loxP* leukemic mice

Haematoxylin- and eosin-stained sections of tissues from leukemic mice (*Mll; Enl; Cre*) were compared with control tissue from an *Mll-Enl* only, age-matched mouse (wt). All organs examined were heavily infiltrated with myeloid cells. The liver shows perivascular deposits of myeloid cells and heavy infiltration of the tissue, and similarly, deposits of leukemic cells were found in kidney between the glomerili. The blood vessel in the liver sections illustrate the high number of leukocytes circulating in these mice, compared with a vessel in the control liver. The spleen shows loss of normal architecture, with myeloid cells in abundance.

would be the nearest to a direct recapitulation of the de novo occurrence of human cancer-associated chromosomal translocations. The Cre-*loxP* recombination system has been shown to facilitate de novo chromosomal translocations in mice (Buchholz et al., 2000; Collins et al., 2000) but no tumor incidence was reported in these initial translocator mice, possibly due to the restricted expression characteristics of the Cre recombinase in these first generation models. We have established a line of mice in which Cre recombinase is expressed via the hematopoietic *Lmo2* gene (Warren et al., 1994) and where *loxP* sites have been engineered into *Mll* and *Enl* loci. We demonstrate that Cre-*loxP*-mediated interchromosomal recombination between the *Mll* and *Enl* genes creates reciprocal chromosomal translocations, which rapidly cause myeloid tumors with rapid onset and high penetrance of leukemogenesis. This approach is a direct recapitulation of human cancer-associated translocations, formally showing that these cause cancers, which are most likely clonal in origin. The strategy can be used to generate de novo reciprocal translocations that effectively recapitulate any naturally occurring translocation in human cancers.

Table 1. Differential leukocyte count from blood smears of *Mll-Enl-Cre* mouse

	Leukocytes (n = 438)	Myeloid cells (n = 417)
Neutrophils	275 (63%)	275 (66%)
Nonsegmented forms	31 (7%)	31 (7%)
Monocytes	20 (4%)	20 (5%)
Myelocytes	61 (14%)	61 (15%)
Myeloblasts	30 (7%)	30 (7%)
Lymphocytes	21 (5%)	

Blood films were prepared at time of sacrifice and stained with May-Grunwald-Giemsa (MGG) stain. Morphology of leukocytes was microscopically examined and assessment of cell type and diagnosis made by reference to published criteria (Bain, 1993; Kogan et al., 2002). Data shown are for a typical distribution profile of cell types.

Results

Interchromosomal translocations between mouse *Mll* and *Enl* genes

The strategy used to generate chromosomal translocations was to introduce *loxP* sites into equivalent introns of the *Mll* and *Enl* genes to those involved in human leukemia translocations, using homologous recombination in ES cells. The ES cells were used to make mice carrying these genetic alterations and inter-bred with Cre-expressing mice. We have previously described the mouse *Mll-loxP* gene in ES cells, at a site corresponding to human translocations (Figure 1A) (Collins et al., 2000). Similarly, a *loxP* site was engineered into an *Enl* gene intron, upstream of exon 2 (Figure 1B). Somatic recombination between these sites should create a mouse fusion gene equivalent to the *MLL-ENL* fusion found in human leukemias with t(11;19) (Ayton and Cleary, 2001).

Initial tests were performed to confirm that translocations were possible between mouse chromosomes 9 and 17 (*Mll* and *Enl* chromosomes, respectively) by expressing Cre protein in ES cells carrying both the *Mll* and *Enl loxP* alleles. After transient expression of Cre protein, genomic DNA and mRNA were iso-

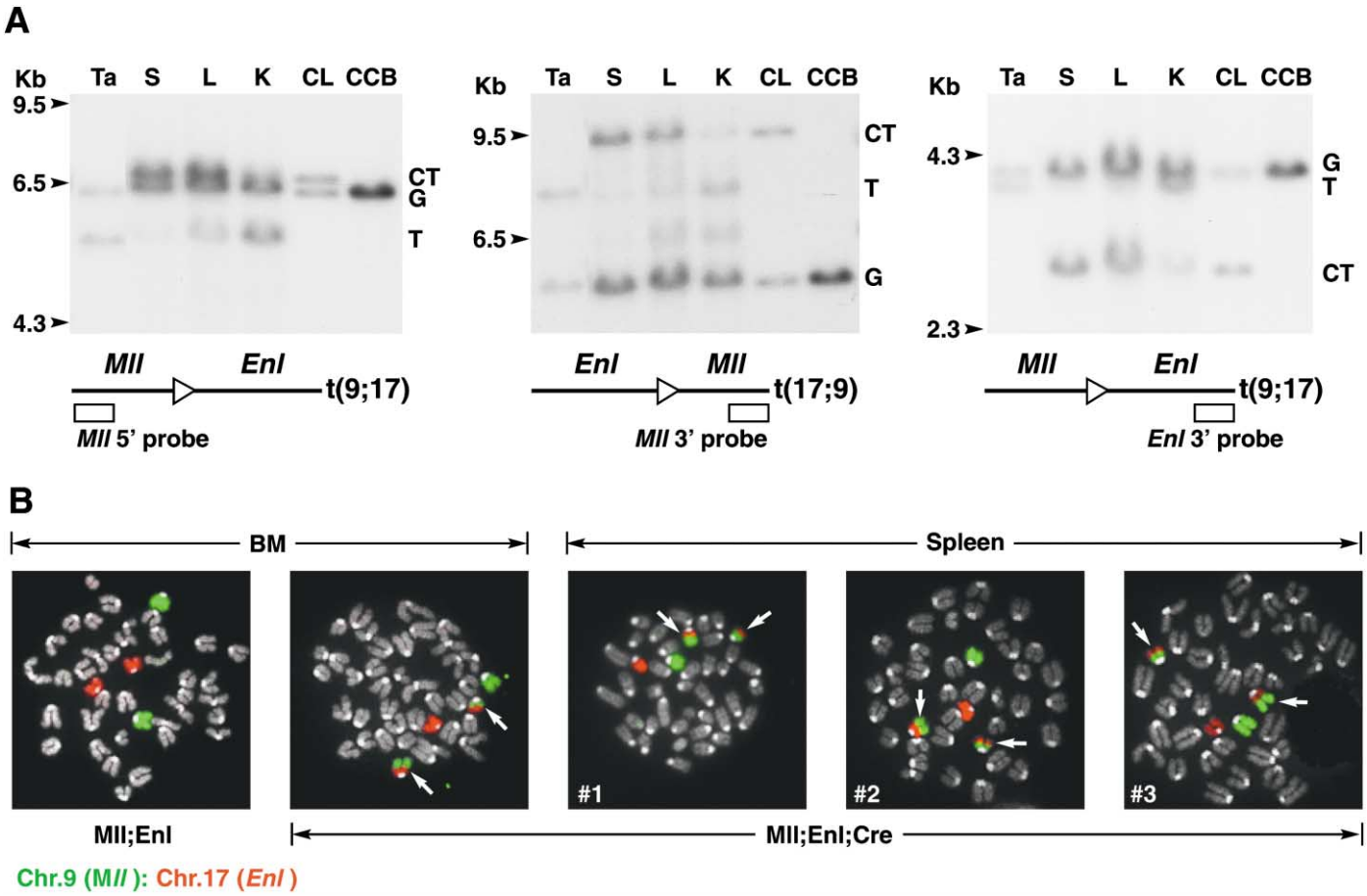

Figure 4. Reciprocal chromosomal translocations occur in *Mll-loxP;Enl-loxP; Cre* mice

A: DNA was prepared from tissues of mice at time of death and filter hybridization analysis carried out using probes able to detect DNA fragments from putative chromosomal translocations. An example (specimen 1) is shown in **A**. Genomic DNAs from the indicated sources were digested with SphI, fragments separated on 0.8% agarose, transferred to nylon membranes and hybridized with an *Mll* 5′ probe (Collins et al., 2000) (left hand panel), digested with KpnI and hybridized with an *Mll* 3′ probe (Collins et al., 2000) (middle panel), or digested with HindIII and hybridized with an *Enl* 3′ probe (left hand panel). Ta = tail biopsy DNA; S = spleen DNA; L = liver DNA; K = kidney DNA; CL = an *Mll-loxP; Enl-loxP; Cre* cell line DNA; CCB = ES cell DNA
Markers were λDNA cut with HindIII (Kb = kilobases).

B: Fluorescence in situ hybridization of cells from bone marrow (BM) or spleen cells of *Mll-loxP; Enl-loxP* or *Mll-loxP; Enl-loxP; Cre* mice using paints for chromosome 9 (FITC) or chromosome 17 (Cy3). FISH analysis of BM from a *Mll-loxP; Enl-loxP* mouse or a leukemic mouse. The FISH analysis of cell lines established from spleen cells of three independent tumors which arose in *Mll-loxP; Enl-loxP; Cre* mice. Representative metaphases are shown. White arrows indicate the translocated chromosomes.

lated and recombination products were analyzed by genomic PCR or RNA-based RT-PCR (Figure 1C). A genomic PCR product, spanning the *Mll-Enl* chromosome t(9;17) junction, was obtained which had the predicted sequence comprising chromosome 9-*loxP* site-chromosome 17 (Figure 1D) and the cells were expressing a fusion mRNA, comprising *Mll* exon 10 fused to *Enl* exon 2 (Figure 1E). These data confirm that the t(9;17) *Mll-Enl* chromosomal translocation can occur in mouse cells and that the fusion mRNA is transcribed.

Myeloid leukemias carrying t(9;17) develop *Mll-Enl-Cre* in mice

The possible tumorigenic effect of the *Mll-Enl* chromosomal translocation in mice was determined by studying mice in which recombination between the *Mll* and *Enl* genes was mediated by Cre recombinase expressed from a knockin of *Cre* into the hematopoietic regulator *Lmo2* (unpublished data). A cohort of 21 mice was generated carrying the *Mll-loxP* and *Enl-loxP* alleles together with the *Lmo2-Cre* allele. Mice carrying the two *loxP* alleles and the *Cre* allele (*Mll-loxP; Enl-loxP; Cre*) were compared with mice carrying only the *loxP* alleles (*Mll-loxP; Enl-loxP*). By 120 days, 100% of the *Mll-loxP; Enl-loxP; Cre* mice had died or been sacrificed due to ill health, while all *Mll-loxP; Enl-loxP* mice remained healthy (Figure 2A; a similar cohort of *Lmo2-Cre*-only mice also remained disease free, data not shown). Post-mortem examination of *Mll-loxP; Enl-loxP; Cre* mice showed splenomegaly, pale livers, kidneys, and bone marrows, but generally no macroscopic abnormality of thymus. Invasion of spleen was observed, with disruption of normal architecture by leukemic cells (Figure 3 compares tissue histology of a normal *Mll-loxP; Enl-loxP* mouse with a leukemic *Mll-loxP; Enl-loxP*; *Cre* mouse). There was extensive infiltration of tumor leukocytes into peripheral organs such as liver and kidneys with prominent

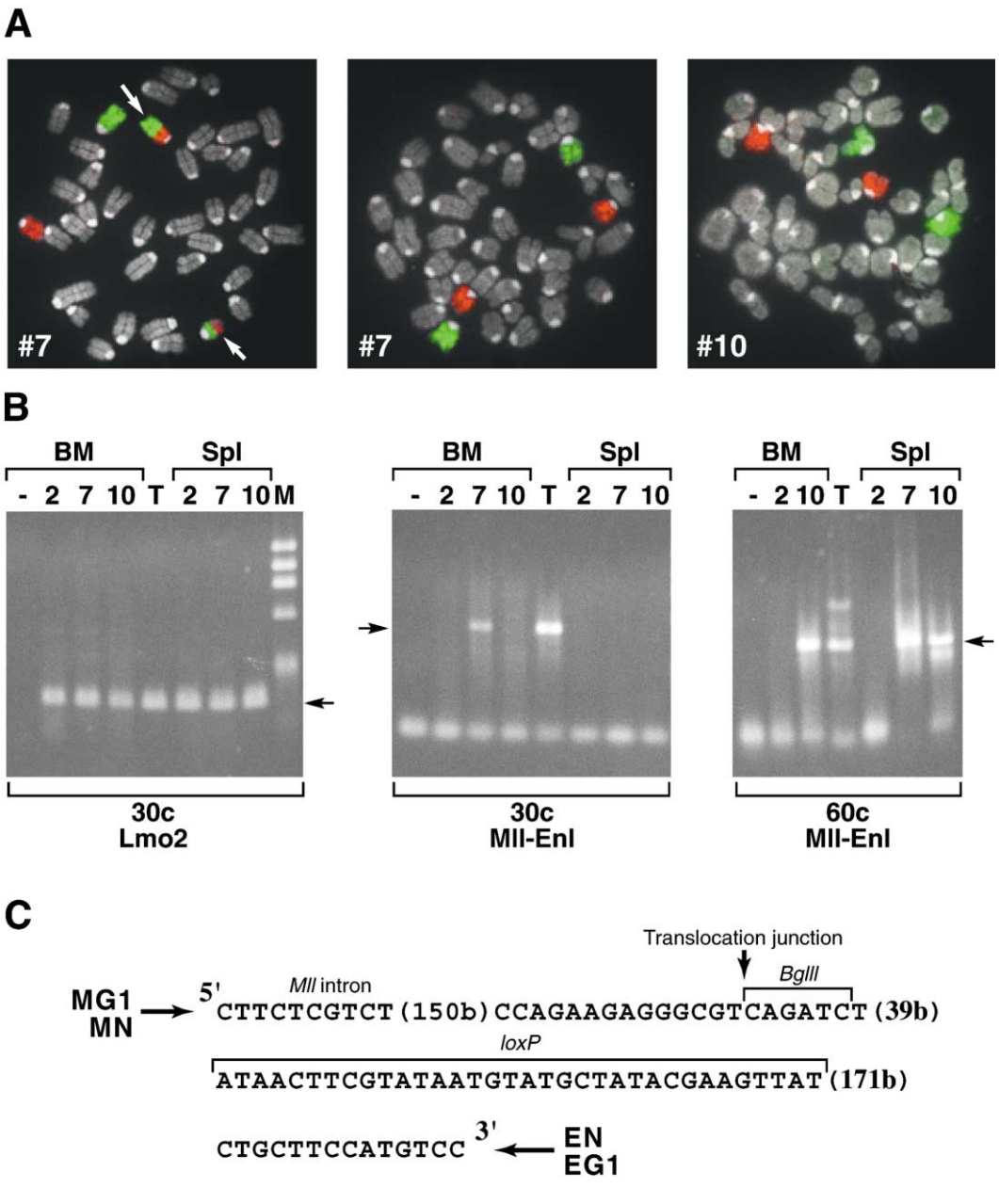

Figure 5. Chromosomal translocations can be detected in young asymptomatic mice

A 12-day-old litter from a cross between *Mll-loxP; Enl-loxP* homozygous mice and heterozygous *Lmo2-Cre* mice was used as a source of cells to examine the occurrence of chromosomal translocations. Pups 2, 7, and 10 were analyzed in detail with fluorescence in situ hybridization and genomic PCR to detect translocation products.

A: Bone marrow cells were maintained in temporary culture and metaphase spreads prepared for FISH with chromosome 9 and 17 paints. Metaphases from pups 2, 7, and 10 were analyzed and representative hybridizations are shown. *Mll-loxP; Enl-loxP; Cre* pup 7 showed metaphases with reciprocal chromosomal translocations (pup 7, left hand panel, representative of 90% of the spreads) or normal metaphases (pup 7, middle panel, 10% of spreads). *Mll-loxP; Enl-loxP; Cre* pup 10 showed only normal metaphases (right hand panel). White arrows mark the position of the translocated chromosomes from pup 7.

B: Genomic DNA was isolated from BM and spleen from pups 2, 7, and 10 and PCR carried out for 30 cycles with *Lmo2*-specific primers (left hand panel), with *Mll* and *Enl* genomic primers (MG1 + EG1) for 30 cycles (middle panel), or nested *Mll* and *Enl* genomic primers (MN + EN) for 30 cycles (right hand panel) to give total of 60 cycles. Pup 2 was a Cre negative (*Mll-loxP; Enl-loxP*) while pups 7 and 10 were *Mll-loxP; Enl-loxP; Cre* mice.

- = no template control; BM = bone marrow; Spl = spleen; T = tumor from an *Mll-loxP; Enl-loxP; Cre* mouse; arrows indicate the PCR band corresponding to the *Lmo2* genomic product or the translocation junction product. White arrows indicate the translocated chromosomes.

C: Sequence of the BM chromosomal translocation product from a tumor.

perivascular deposits of tumor cells in liver and in between glomerili of kidney (Figure 3).

Blood smears showed large numbers of circulating myeloid cells (Figure 2B), with a mixture of immature myeloblasts and more mature myeloid forms. Microscopic analysis of the different morphological cell types present in the leukemias is documented in Table 1. Ninety-five percent of the leukocytes are nonlymphoid cells, and of these, around 20% are immature cells/blasts and around 80% are mature myeloid cells, comprising around 60%–70% neutrophils, around 10% nonsegmented forms, and 5% monocytic (Table 1). The surface antigen phenotype of bone marrow, spleen, or thymus cells of tumor mice showed that the *Mll-loxP; Enl-loxP; Cre* mice develop a leukemia characterized by expression of the myeloid markers Mac-1 (CD11b) and Gr-1 (Ly-6G) (Figure 2C). Cell lines established from spleens of leukemic mice also have a Mac-1; Gr-1 phenotype, similar to those found on the primary biopsy material (Figure 2C). Neither the primary cells nor the cell lines express the progenitor markers Sca-1 or c-Kit, nor do they express the lymphoid markers CD4, CD8, or B220. The cells were initially grown in vitro in serum with added growth factors and subsequently grown without added growth factors. The myeloid markers are similar to cultured cells from leukemic *Mll-AF9* knockin mice (Dobson et al., 1999) (Figure 2C). We conclude that the *Mll-loxP; Enl-loxP; Cre* mice develop a leukemia involving myeloid lineage. All the *Mll-loxP; Enl-loxP; Cre* mice succumb to the myeloid neoplasm with aggressive infiltration of myeloid cells into various organs. Overall, the morphological data, surface phenotype, and tissue involvement are most consistent with the classification of the leukemia as "myeloproliferative-disease-like myeloid leukemia" according to mouse leukemia classification (Kogan et al., 2002).

The leukemia observed in the *Mll-Enl* mice causes death in all cases, unless the mouse was sacrificed due to obvious ill heath, and *Mll-loxP; Enl-loxP; Cre* mice all succumb in the first 120 days after birth. The disease was only observed in individuals with both the *loxP* alleles and expressing Cre recombinase. This suggested that the cause of leukemia was the generation of cells in the individual mice with chromosomal translocations which outgrow and present as overt leukemia. The leukemic cells were not transplantable in nonirradiated recipients, although transplantation per se does not serve as a defining element for mouse hematopoietic neoplasm (Kogan et al., 2002). The presence of the reciprocal chromosomal translocations in mice which succumbed to leukemia was confirmed by analysis of genomic DNA and by direct observation of translocated chromosomes. The presence of translocations in the leukemias was shown using filter hybridization of DNA from tumor cells. DNA probes from 5′ or 3′ of the *Mll* gene breakpoint or from 3′ of the *Enl* breakpoint were used to examine rearranged genomic DNA restriction fragments (Figure 4A). This substantiated the presence of reciprocal translocations in the mice since the anticipated restriction fragment products of translocations were found. These were, specifically, a 7.0 Kb SphI fragment detected by the 5′ *Mll* probe, a 9.5 Kb KpnI fragment detected by the 3′ *Mll* probe, and a 3.0 Kb HindIII fragment detected by the 3′ *Enl* probe. Formal confirmation of the genomic breakpoint of the translocation was obtained by genomic PCR of a product spanning relevant *Mll* and *Enl* introns, followed by DNA sequencing. The presence of the translocated *Mll* DNA fragment was confirmed in all the leukemic mice in the cohort (data not shown).

Table 2. Modal distribution of chromosomes in metaphase spreads of cell lines from *Mll-Enl-Cre* mice

Cell line	No. metaphases	No. chromosomes	Modal number
1	18	37 (n = 1) 39 (n = 3) 40 (n = 12) 41 (n = 2)	39.8
2	18	39 (n = 2) 40 (n = 14) 41 (n = 2)	40.0
3	19	36 (n = 2) 40 (n = 15) 41 (n = 2)	39.7

Spleen cells were established in culture from tumour-bearing mice and metaphase chromosomes prepared after treatment of the cells with colcemid. Chromosome spreads were made from fixed nuclei and spread on glass slides for manual counting.

The cloned PCR product from *Mll-loxP; Enl-loxP; Cre* mouse tumor cells confirmed the site-specific joining of *Mll* and *Enl* introns (Figure 5C), which was identical to that of the ES cell product and showed site-specific recombination between the *loxP* sites.

A direct visualization of the reciprocal translocations was obtained by fluorescence in situ hybridization (FISH) of tumor cell chromosomes from the *Mll-loxP; Enl-loxP; Cre* mice compared with *Mll-loxP; Enl-loxP* mice (Figure 4B). Short term bone marrow cultures were used for the preparation of metaphase chromosomes. This revealed reciprocal chromosomal translocations by hybridization with chromosome 9 and chromosome 17 paints (Figure 4B). In addition, metaphases from *Mll-loxP; Enl-loxP; Cre* tumor cell lines obtained from spleen cells also had reciprocal translocations t(9;17) and t(17;9) (Figure 4B). The tumors from the *Mll-loxP; Enl-loxP; Cre* mice therefore carry reciprocal chromosomal translocations. All the mice in the *Mll-loxP; Enl-loxP; Cre* cohort were shown to have *Mll*-chromosomal translocations by either filter hybridization or by FISH analysis. In addition, karyotypes associated with these de novo tumors remained euploid, as at least the modal chromosome number of three independent cell lines, derived from leukemic mice, were normal (i.e., 40 chromosomes; Table 2).

Chromosomal translocations occur early in *Mll-Enl* mice

The youngest *Mll-loxP; Enl-loxP; Cre* mouse to develop leukemia was about 1.5 months of age. Thus, the onset of disease in these *Mll-Enl* translocator mice occurred very early in life and with a high penetrance, and was always associated with the presence of the chromosomal translocations. Therefore, the chromosomal translocations events between *Mll* and *Enl* must occur early in the *Mll-Enl-Cre* mice to allow time for disease manifestation. Possible timing and frequency of translocations were obtained using FISH on chromosomes from bone marrow cells of young mice (12 days of age). Six *Mll-loxP; Enl-loxP; Cre* pups from one litter (of 12) were analyzed. One showed translocations by FISH analysis in all metaphases examined (Figure 5A, pup 7, 23 metaphases examined) while the other five pups had only normal chromosomes in 20-23 metaphases examined (Figure 5A; a representative metaphase from pup 10).

Even though we obtained differential results with detection of chromosomal translocations in the 12 day mice, the early

onset of leukemia in the cohort (Figure 2) was suggestive of early and frequent interchromosomal translocations, followed by selection of the cell(s) carrying the aberrant chromosomes. The sensitivity of the analysis for the 12 day pup 7 and pup 10 littermates was, therefore, extended using genomic PCR to detect the junction of the chromosomal translocation t(9;17). DNA was prepared from bone marrow (BM; Figure 5B) or spleen cells and splenic DNA from a leukemic mouse, and quality control was carried out using an *Lmo2* gene primer pair. This yielded an *Lmo2* product at 30 PCR cycles in all samples. When the *Mll-Enl* chromosomal junction PCR was assessed at 30 cycles, we detected a product in the DNA of pup 7 bone marrow and the tumor DNA but not in bone marrow DNA of pup 10 nor in either pups 7 or 10 spleen DNA (Figure 5B, central panel). No product was obtained with DNA from pup 2, which was *Mll-loxP; Enl-loxP* only. The sequence of the tumor PCR product was obtained to confirm the specific junctional information (Figure 5C).

The presence of low concentrations of chromosomal translocation-positive cells was assessed in these samples using nested PCR, carried out for a further 30 PCR cycles. In this analysis, we observed translocation-specific PCR products from bone marrow DNA from pup 10 (pup 7 shows a product at 30 cycles) and from spleen DNA of both pups 7 and 10 (Figure 5B), which did not amplify a product at 30 cycles. No product was obtained from the Cre-negative pup 2. These data therefore show that *Mll-Enl* chromosomal translocations can occur in young mice, and that in some cases at least, it appears that these arise initially in cells of bone marrow origin and subsequently these cells migrate to spleen.

Discussion

Early onset of Mll-Enl-associated leukemia

As in humans, the chromosomal translocation between mouse *Mll* and *Enl* results in leukemia. The data from our study show that mice with the compound genotype comprising *loxP* recombination sites with *Mll* and *Enl* genes develop leukemia only if Cre recombinase is expressed. The *Mll-Enl* chromosomal translocation thus always results in malignancy. The onset of malignancy is rapid, which is consistent with retroviral transduction studies (Ayton and Cleary, 2001; Lavau et al., 1997; Slany et al., 1998), but in the translocator mice, the development of disease occurs after a somatic event (i.e., chromosomal translocation) and is not due to the simultaneous transplantation of many transduced, Mll-Enl-expressing cells. This implies that the effect of Mll-Enl is paramount in the leukemic process, perhaps the only event needed for leukemia. It is significant that young mice with low number of cells with chromosomal translocations (i.e., only detectable by nested PCR) coexist with littermates with high numbers of chromosomal translocation-positive cells. This must reflect the time at which the interchromosomal event occurred (these are not a constitutional chromosomal translocation, and we can define neither the timing in individual mice nor how long overt disease takes to manifest itself after the chromosomal translocation event) and the putative selectivity which is conferred on the cell that undergoes chromosomal translocation. The early appearance of large proportions of translocation carrying cells (i.e., as early as 12 days) further could suggest that the emergence of overt hematopoietic malignancy is wholly dependent on the *Mll-Enl* fusion gene, without the intervention of further mutational events, as has been suggested for human *MLL* translocations (Ford et al., 1993). A refinement of this argument will depend on a detailed study of candidate gene mutation and of genetic analysis for chromosomal instability. At present, we have examined the ploidy of three cell lines obtained from the tumor-bearing mice, and these exhibit normal chromosome numbers (Table 2).

The *Lmo2* gene is needed for the development of adult hematopoiesis (Yamada et al., 1998), and Cre expression from the *Lmo2-Cre* knockin gene occurs in bone marrow (unpublished data). The major site of the chromosomal translocation is therefore likely to be bone marrow cells; this is supported by the PCR and FISH analysis of 12-day-old mice (Figure 5) where concentrations of cells with chromosomal translocations are high in bone marrow but not in spleen. The leukemia in this model may initiate in bone marrow progenitors, which progress and migrate to remote locations such as spleen, but this cannot be proved in this de novo model. At the time of overt disease, the abnormal myeloid cells are found to be invading somatic tissues with typical, large perivascular deposits in liver and kidney.

De novo chromosomal translocation mouse models

The de novo chromosomal translocations in mice are the closest to natural, human chromosomal translocations of any model thus far. The finding that interchromosomal translocations between *Mll* and *Enl* in mice invariably lead to leukemia suggests that the Cre-*loxP* in vivo approach (Buchholz et al., 2000; Collins et al., 2000) can be employed, with appropriately specific Cre-expressing mice, to any pair of genes as long as the orientation to the centromere does not result in dicentric aberrant chromosomes. The employment of cell-specific Cre expression or inducible Cre will increase the versatility of this approach and potentially make experiments possible which evaluate hierarchies of genetic changes associated with human cancers with chromosomal translocations. In addition, Cre-dependent, interchromosomal translocations result in leukemias with reciprocal translocated chromosomes, which is a unique feature of this approach, and which may well be a crucial issue in cases where the involvement of both translocated chromosomes has been implicated (He et al., 1997).

The translocator mouse approach should not only provide the basis for generating chromosomal translocations to mimic the diversity of breakpoints in leukemias and sarcomas but, in addition, should provide an experimental framework for exploring the role of translocations in epithelial tumors, where recurrent and non-recurrent translocations are found (Mitelman et al., 2000). While tumors of epithelial cell origin do have chromosomal translocations (Dutrillaux, 1998), they do not appear to fall into the recurrent category found in leukemias and sarcomas. Whether these arise due to genetic instability alone or whether they have, in addition, pathogenic consequences for the individual tumors (idiopathic) is not known. The ability to make de novo translocations to recapitulate idiopathic chromosomal translocations should be an invaluable tool to ascertain the significance of these aberrant chromosomes in cancer.

Experimental procedures

Gene targeting and production of translocator mice

The mouse *Mll* genomic fragment with exon 10 and *loxP* has been previously described (Collins et al., 2000). Genomic λ phage DNA clones of the mouse

Enl gene were isolated from a library of 129 DNA using a human cDNA clone made by PCR amplifying a 350 bp fragment including exon 2. The *loxP*-hygromycin and puromycin-*loxP* cassettes (Collins et al., 2000) were cloned into BglII (Collins et al., 2000) and SphI sites of *Mll* and *Enl*, respectively, to generate the two targeting vectors pMll-loxP-hygro and pEnl-loxp-puro (Figures 1A and 1B). Homologous recombination was carried out into CCB ES cells as described (Robertson, 1987) using either hygromycin- or puromycin-resistant mouse embryonic fibroblast feeders as appropriate (Johnson et al., 1995; Linnell et al., 2001). Targeted ES clones were identified by filter hybridization (LeFranc et al., 1986) using 5′ and 3′ flanking probes from *Mll* genomic DNA (Figure 1A). The 5′ *Enl* flanking probe was a 1 kb BamHI + XbaI fragment located 5′ of exon 2 (not shown) and the 3′ probe was a KpnI-SphI fragment (Figure 1B). A clone with targeted *Mll* was retransfected with the pEnl-loxP-puro clone to make double-targeted ES clones. To determine if interchromosomal translocation between *Mll* and *Enl* could occur, double-targeted clones were transiently transfected with the Cre recombinase expression vector pPGKCrebpA using electroporation (Collins et al., 2000). After 72 hr, cells were harvested, RNA was prepared using Trizol (Sigma), and DNA using a standard phenol-chloroform procedure. The presence of the *Mll-Enl* translocation chromosome t(9;17) was confirmed by genomic PCR using 0.5 μg DNA in a PCR reaction with 5 ng primers MG1 + EG1 in the following conditions: initial 95°C 2′, 95°C 30 s touchdown (Feinberg and Vogelstein, 1983) 65°–56°C, 2 cycles each for 30 s, 10 cycles at 55°C and extend 72°C for 1′ with final 10′ extension. A PCR product of 470 bp was visualized on 1.3% agarose gels and cloned into pGEM-T (Promega) for sequencing (Figure 1D). RT-PCR was carried out with cDNA synthesized using the ES cell RNA. cDNA was synthesized with an oligo-dT primer using 5 μg total RNA in final volume of 100 μl. One mircroliter of cDNA was amplified in a 25 μl PCR reaction containing 5 ng of primers MR1 + ER1 (Figure 1E) in the following conditions: 95°C 2′, 35 cycles of 95°C for 30 s, 60°C for 1′, 72°C for 1′, then 1 μl was nested for a further 30 cycles. A PCR product of 400 bp was visualized on 1.3% agarose gels and cloned into pGEM-T (Promega) for sequencing.

Phenotypic analysis

Mice were strictly monitored and were sacrificed at the first signs of ill health. Blood smears were prepared at time of death and stained with May-Grunwald-Giemsa (MGG) stain. Full post-mortem examination was carried out and tissues fixed in 10% buffered formalin. After wax embedding, 4 μM sections were made and stained with haematoxylin and eosin. Blood films and sections were analyzed and images made with a Zeiss Axioplan microscope. Fluorescence activated cell sorter analysis was carried out on a FACSCalibur and data collected using Cellquest software (BD). Antibodies used at 1/10 dilution in PBS were directly coupled to the respective fluorochrome (Pharmingen). Antibodies used were PE-labeled anti-Mac-1 (CD11b), FITC -labeled anti-Gr-1 (Ly-6G), FITC-labeled anti-CD4 (L3T4), PE-labeled anti-CD8a (Ly-2), FITC-labeled anti-B220 (CD45R), PE-labeled anti-Sca-1, and FITC-labeled anti-c-kit, purchased from Pharmingen; BD Biosciences. Spleen and bone marrow cells were prepared as single cell suspensions by passing through a 70 μM cell strainer (BD), washed in cold PBS, and suspended to a concentration of $<5 \times 10^7$cells/ml in cold PBS/1%FCS. Antibody dilutions were added to 100 μl of cells and incubated on ice for 1 hr. The appropriate isotype controls were used for each antibody.

Determination of chromosomal translocations

Tumour cells (spleen or bone marrow) were prepared as single cell suspensions and cultured in 5% CO_2 in RPMI medium supplemented with 20% fetal calf serum, 20 u/ml IL2, 10 u/ml IL6, IL7, and GM-CSF (Roche), 5% WEHI231 confluent culture supernatant (IL3 source [Karasuyama and Melchers, 1988]) plus 100 μg/ml penicillin-streptomycin and 200 μg/ml gentamycin. For primary cultures, medium was replenished as necessary and nonadherent cells retained for further use. After initial passages, cells were transferred to 10% concentrations of interleukins and subsequently grown on in RPMI medium supplemented with 10% fetal calf serum without additional added growth factors. Fluorescence in situ hybridization (FISH) was carried out with whole chromosome paints according to the manufacturer's instructions (Cambio, Cambridge, United Kingdom). In outline, metaphases were prepared by culturing tumor cells for 4 hr in the presence of 0.2 μg/ml colcemid (Invitrogen). Cells were harvested and resuspended in hypotonic (75 mM) KCl and incubated for 30 min at 37°C. Nuclei were washed twice in fixative (3:1 ethanol: glacial acetic acid), resuspended in fixative, and dropped onto glass slides. After denaturation with 70% formamide, metaphase spreads were hybridized with FITC-labeled chromosome 9 paint and Cy3-labeled chromosome 17 paint for 16 hr at 42°C. After two stringent washes with 50% formamide, spreads were mounted with DAPI (Vector Laboratories, Burlingame, California) and analyzed at 1000× magnification under oil immersion. Image analysis was performed with Smart Capture 2.1 (Digital Scientific, Cambridge, United Kingdom). Bone marrow and spleen single cell suspensions were short-term cultured, as for tumor cells for 2–5 days prior to FISH analysis.

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